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(21) International Application Number: PCT/US98/11391 (22) International Filing Date: 5 June 1998 (05.06.98) (30) Priority Data: 60/048,793 6 June 1997 (06.06.97) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; Office of Technology Transfer, Fifth floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US). (72) Inventors: RAY, Eyal; 7965 Camino Huerta, San Diego, CA 92122 (US). ROMAN, Mark; 8742-33 Villa La Jolla Drive, La Jolla, CA 92037 (US). (74) Agent: TAYLOR, Stacy, L.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: INHIBITORS OF DNA IMMUNOSTIMULATORY SEQUENCE ACTIVITY (57) Abstract The invention consists of oligonucleotides which inhibit the immunostimulatory activity of ISS-ODN (immunostimulatory sequence oligodeoxynucleotides) as well as methods for their identification and use. The oligonucleotides of the invention are useful in controlling therapeutically intended ISS-ODN adjuvant activity as well as undesired ISS-ODN activity exerted by recombinant expression vectors, such as those used for gene therapy and gene immunization. The oligonucleotides of the invention also have anti-inflammatory activity useful in reducing inflammation in response to infection of a host with ISS-ODN containing microbes, in controlling autoimmune disease and in boosting host Th2 type immune responses to an antigen. The invention also encompasses pharmaceutically useful conjugates of the oligonucleotides of the invention (including conjugate partners such as antigens and antibodies).		

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INHIBITORS OF DNA IMMUNOSTIMULATORY SEQUENCE ACTIVITY

BACKGROUND FOR THE INVENTION5 1. *Field of the Invention*

The invention relates to immunostimulatory sequences in DNA. The invention further relates to recombinant expression vectors for use in gene therapy.

2. *History of the Related Art*

10 Recombinant expression vectors are the tools which researchers and clinicians use to achieve the goals of gene therapy and gene immunization. In gene therapy, viral and non-viral vectors are used to deliver an expressible gene into a host to replace a missing or defective gene, or to otherwise supply the host with a therapeutically beneficial polypeptide. In gene immunization, mostly non-viral vectors are used to induce an immune response by the host to an expressed antigen.

15

One of the obstacles to successful clinical practice of both gene therapy and gene immunization has been the often transient nature of the gene expression achieved *in vivo*. Transient gene expression is less problematic in gene immunization, where immune responses sufficient for certain immunization schemes may be stimulated by even short-
20 term exposure to expressed antigen. In addition, several options are available to boost the host immune response to antigen, including use of the vector itself as an adjuvant for the desired immune response by exposing the host to non-coding, immunostimulatory nucleotide sequences (ISS-ODN) present in the vector (Sato, *et al.*, *Science*, 273:352-354 (1996)).

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However, in a gene therapy protocol, premature loss of gene expression deprives the host of the potential benefits of the therapy (Friedmann, *Scientific American*, "Making Gene Therapy Work" (June 1997)). Repetitive dosing to extend exposure of the host to a therapeutic polypeptide can require that different vectors be used to deliver each dose so
5 the host immune response to vector antigens is minimized (Tripathy, *et al.*, *Nature Med.*, 2:545-550 (1996)).

One potential source of vector immunogenicity are ISS-ODN in the genome of the microbial species used to construct recombinant expression vectors. To explain, the CpG motifs which characterize ISS-ODN are present in bacteria and viruses (including
10 retroviruses) at a much greater frequency than is seen in vertebrate genomes. One consequence of ISS-ODN activity is the ISS-ODN induced production of cytokines such as interferon- α (INF- α), INF- γ and interleukin-12 (IL-12). This ISS-ODN induced inflammation is believed to be defensive against microbial infection in vertebrates and is also believed to be produced in response to ISS-ODN introduced into a host as
15 oligonucleotides or as part of a recombinant expression vector.

SUMMARY OF THE INVENTION

The invention provides compounds consisting of oligodeoxynucleotides, ribonucleotides or analogs thereof which specifically inhibit the immunostimulatory activity of ISS-ODN.

20 ISS-ODN induced secretion of INF- α in particular can suppress recombinant gene expression and directly impedes mRNA and protein synthesis in transfected cells. Thus, inhibition of ISS-ODN activity substantially avoids ISS-ODN induced loss of gene expression, thereby prolonging the availability of the expressed polypeptide to a host undergoing gene therapy or gene immunization with an ISS-ODN containing recombi-
25 nant expression vector. Further, the need for repetitive dosing to prolong availability of expressed proteins and for extensive reengineering of recombinant expression vectors

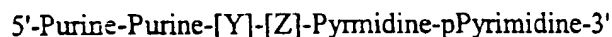
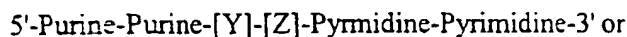
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to eliminate ISS-ODN sequences is avoided through use of the compounds of the invention.

The compounds of the invention are also useful in modulating the immunostimulatory activity of ISS-ODN administered as adjuvants to boost host immune responses to
5 antigen in, for example, immunotherapy. In this respect, the compounds of the invention permit exquisite control over the effect of ISS-ODN based adjuvants in a host.

Further, the compounds of the invention reduce host inflammation generated in response to an infection by an ISS-ODN containing bacteria or virus. Advantageously, the compounds of the invention can be administered to inhibit ISS-ODN activity exerted by
10 a microbe even if the identity of the particular ISS-ODN present in the microbe is unknown. Thus, the compounds of the invention can be considered to be broad spectrum anti-inflammatory agents.

In one aspect, the ISS-ODN inhibitory compounds of the invention are synthesized oligonucleotides (I-ON) which are comprised of the following general primary
15 structures:



where Y is any naturally occurring or synthetic nucleotide except cytosine and is preferably guanosine or inosine (for RNA I-ON). In general, Z is any naturally occurring
20 or synthetic nucleotide or repeat of the same nucleotide. Preferably, when Y is inosine, Z is inosine or one or more guanosine(s). Where Y is guanosine, Z is preferably guanosine or one or more unmethylated cytosine(s). However, when Y is not guanosine or inosine, Z is guanosine or inosine. Most preferably, the 5' purines are the same nucleotide, as are the 3' pyrimidines. For example, where ** is YZ, the 5' purines and

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3' pyrimidines may be AA**TT, AG**TT, GA**TT, GG**TT, AA**TC, AG**TC, and so forth. Any sequences present which flank the hexamer core sequence are constructed to substantially match the flanking sequences present in any known ISS-ODN.

Inhibitory I-ON of the invention are prepared in a pharmaceutically acceptable composition for use in a host. I-ON may be mixed into the composition singly, in multiple copies or in a cocktail of different I-ON. Alternatively, the inhibitory I-ON of the invention may be incorporated into a recombinant expression vector. The inhibitory I-ON can also be provided in the form of a kit, comprising inhibitory I-ON and recombinant expression vector constructs which contain, or are susceptible to insertion of, a gene of interest.

A particular advantage of the I-ON of the invention is that they can be used to target ISS-ODN in any ISS-ODN containing recombinant expression vector or microbe, whether or not the nucleotide composition of the vector or microbe is known. Indeed, it is not necessary that the existence, identity or location of ISS-ODN in the vector or microbe be known. If ISS-ODN are not present in the vector or microbe, the I-ON of the invention will simply have no effect. However, if ISS-ODN are present in the vector or microbe, it can be expected that their immunostimulatory activity will be inhibited in a dose-dependent manner by the I-ON even if the specific structure or location of the ISS-ODN in the vector or microbe is not known.

Thus, in another aspect, the invention provides a simple and effective alternative to the arduous task of eliminating ISS-ODN activity from recombinant expression vectors by identifying all ISS-ODN present in the vector and removing them.

Further in this regard, the invention provides methods for screening recombinant expression vectors for the presence of ISS-ODN and for identifying additional inhibitory I-ON. In the former respect, the presence of ISS-ODN in a recombinant expression vector is confirmed by incubating the vector in a population of lymphocytes with an I-

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ON of known inhibitory activity and comparing the difference, if any, in the level of ISS-induced cytokine production by the lymphocytes before and after I-ON incubation.

In the latter respect, additional inhibitory I-ON having the characteristics disclosed herein are identified by their ability to inhibit the immunostimulatory activity of a known

5 ISS-containing polynucleotide or recombinant expression vector.

In yet another aspect, the invention further provides a useful anti-inflammatory agent for use in inhibiting the immunostimulatory activity of any ISS-ODN present in an infectious bacterium or virus.

In addition, the invention provides useful means for modulating the immunostimulatory
10 activity of ISS-ODN supplied to a host for immunostimulation (e.g., as an adjuvant).

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a graph which represents *in vivo* inhibition of ISS-ODN immunostimulatory activity by inhibitory I-ON of the invention (I-ON DY1019 and
15 DY1041 (having hexamer regions consisting of, respectively, AAGGTT and AAGCTT)). Lymphocyte proliferation stimulated in a murine model by the ISS-ODN (DY1038, having a hexamer region consisting of AACGTT) was compared in the presence or absence of the I-ON. A decline in measured counts-per-minute (CPM; vertical axis) represents inhibition of ISS-ODN immunostimulatory activity in the Figure. Dosages
20 for each I-ON tested are shown along the horizontal axis. DY1039 (an ISS-ODN with the cytosine), DY1040, DY1042 and DY1043 (all with CC dinucleotides in place of the CG dinucleotide of DY1038) served as controls. To confirm the location of potential competition with DY1038, all of the oligonucleotides were identical to DY1038 except for the hexamer regions identified and DY1043 (an irrelevant sequence control).

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FIGURE 2 is a graph which confirms *in vivo* dose dependent inhibition of ISS-ODN immunostimulatory activity by the DY1019 and DY1041 I-ON of the invention. Lymphocyte proliferation stimulated in a murine model by a different ISS-ODN than the one tested in the experiment of FIGURE 1 (DY1018) was compared in the presence or
5 absence of the I-ON. A decline in measured counts-per-minute (CPM; vertical axis) represents inhibition of ISS-ODN immunostimulatory activity in the Figure. Dosages for each I-ON tested are shown along the horizontal axis. Inhibitory activity of I-ON DY1019 and DY-1041 increased with dosage, with the increase in activity of DY1019 being proportional to the increase in dosage. To confirm the location of potential
10 competition with DY1018, DY1019 and DY1041 are identical to DY1018 except for the hexamer regions identified.

FIGURE 3 is a graph which represents *in vivo* dose dependent inhibition of ISS-ODN immunostimulatory activity by several inhibitory I-ON of the invention. Lymphocyte proliferation stimulated in a murine model by DY1038 was compared in the presence or
15 absence of the I-ON. A decline in measured counts-per-minute (CPM; vertical axis) represents inhibition of ISS-ODN immunostimulatory activity in the Figure. Dosages for each I-ON tested are shown along the horizontal axis. In descending order, the most inhibitory activity was displayed by I-ON DY1019, DY-1041, DY1048, DY1050 and DY1060 (the latter have hexamer regions consisting of, respectively, AGGGTT,
20 GAGGTC and TTGCAA). DY1039 (an ISS-ODN with the cytosine methylated), DY1040 and DY1043 (the latter with CC dinucleotides in place of the CG dinucleotide of DY1038) served as controls. To confirm the location of potential competition with DY1038, all of the oligonucleotides were identical to DY1038 except for the hexamer regions identified and DY1043 (an irrelevant sequence control) .

25 FIGURE 4 is a graph which represents *in vivo* dose dependent inhibition of ISS-ODN immunostimulatory activity by inhibitory I-ON of the invention. INF- γ production stimulated by DY1018 ISS-ODN in a murine model was compared in the presence or absence of the I-ON. A decline in measured INF- γ (vertical axis) represents inhibition

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of ISS-ODN immunostimulatory activity in the Figure. Dosages for each I-ON tested are shown along the horizontal axis. Some inhibitory activity was observed for all but one I-ON, with the most activity being displayed by I-ON DY1019 and DY-1041, as well as DY1042 (having a hexamer region consisting of TTCCTT). The insert separates out
5 the data for inhibition of INF- γ production by DY1019. To confirm the location of potential competition with DY1018, all of the oligonucleotides were identical to DY1018 except for the hexamer regions identified and DY1043 (an irrelevant sequence control)

Figure 5 is a graph which represents the adjuvant properties of IIS-ODN, whereby a Th2-type cellular immune response in antigen (β -galactosidase) immunized mice is induced
10 by coadministration of the antigen and IIS-ODN DY1019 (identified in the Figure as β -gal/M-ODN). TH2 responses are represented by IgE levels measured post-boosting. The values obtained are compared to IgE levels measured in mice immunized with antigen and the ISS-ODN composition b-gal/ISS-ODN (5'-AATTCAACGTTTCGC-3'), pKISS-3 (a plasmid having three copies of the AACGTT ISS-ODN hexamer in the backbone) and
15 pKISS-0 (a plasmid having no copies of the AACGTT ISS-ODN hexamer in the backbone), as well as mice which received only saline. Potent IgE responses (Th2-type responses) above 1000 CPM were obtained only in the mice which received saline (approximately 1200 CPM at 1 week post-boosting) and β -gal/M-ODN (approximately 1750 CPM at 1 week post-boosting).

DETAILED DESCRIPTION OF THE INVENTION

A. Activity and Structure of IIS-ON

1. *IIS-ON Activity and Screening Assay*

The IIS-ON of the invention reduce the immunostimulatory effect of ISS-ODN. Structurally, ISS-ODN are non-coding oligonucleotides 6 mer or greater in length which may include at least one unmethylated CG motif. The relative position of each CG sequence in ISS-ODN with immunostimulatory activity in certain mammalian species (e.g., rodents) is 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position). Many known ISS-ODN flank the CG motif with at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (e.g., TC or TT) to enhance the B lymphocyte stimulatory activity of the immunostimulatory polynucleotide (see, e.g., Krieg, *et al.*, *Nature*, 374:545-549, 1995).

Functionally, ISS-ODN enhance the cellular and humoral immune responses in a host, particularly lymphocyte proliferation and the release of cytokines (including IFN) by host monocytes and natural killer (NK) cells. Bacterial DNA contains unmethylated CpG dinucleotides at a frequency of about one per every 16 bases. These dinucleotides are also present in certain viral species, but are notably underrepresented in vertebrate species.

It is believed that the ability of mycobacteria as well as other bacterial and viral species to stimulate lymphocyte proliferation, IL-12 production, tumor necrosis factor (TNF) production, natural killer (NK) cell activity and IFN- γ secretion is owed to the presence of ISS-ODN in bacterial and viral DNA (see, e.g., Krieg, *Trends in Microbiology*, 4:73-76 (1996)). In contrast, CpG suppression and methylation in vertebrates may be an evolutionary response to the threat of bacterial and viral infection. Interestingly, a CpG containing oligonucleotide comparable to bacterial ISS-ODN has also recently been

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implicated in the onset and exacerbation of autoimmune disease through an IL-12 dependent pathway (Segal, *et al.*, *J.Immunol.*, 158:5087 (1997)).

Immuostimulation by synthetic ISS-ODN *in vivo* occurs by contacting host lymphocytes with, for example, ISS-ODN oligonucleotides, ISS-ODN oligonucleotide-conjugates and
5 ISS-containing recombinant expression vectors (data regarding the activity of ISS-ODN conjugates and ISS-ODN vectors are set forth in co-pending, commonly assigned U.S. patent applications Serial Nos. 60/028,118 and 08/593,554; data from which is incorporated herein by reference solely to demonstrate ISS-ODN immunostimulatory activity *in vivo*). Thus, while native microbial ISS-ODN stimulate the host immune
10 system to respond to infection, synthetic analogs of these ISS-ODN may be useful therapeutically to modulate the host immune response not only to microbial antigens, but also to tumor antigens, allergens and other substances (*id.*).

Although the invention is not limited by any theory regarding the mechanism of action of the IIS-ON, it is believed that they compete with ISS-ODN for binding to the cellular
15 membrane of host lymphocytes. The region of ISS-ODN which confers their immunostimulatory activity is believed to be the 6 mer or greater length of nucleotides which include an unmethylated dinucleotide (e.g., CpG). Therefore, it is believed that the presence of a region of about 6 mer or greater length having at least one competing dinucleotide (defined as [Y]-[Z] and [Y]-poly[Z] in the formulae set forth below) therein
20 confers ISS-inhibitory activity on the IIS-ON of the invention.

Thus, the inhibitory compounds of the invention are synthesized oligonucleotides (IIS-ON) which inhibit the immunostimulatory activity of ISS-ODN in vertebrates and vertebrate immune cells.

To identify IIS-ON from a pool of synthesized candidate IIS-ONs, the following steps
25 provide a simple and efficient means of rapidly screening the candidate pool:

- 10 -

- a. A population of cultured, antigen stimulated lymphocytes and/or monocytes is contacted with an ISS-ODN to induce lymphocyte proliferation, IFN β , IFN- α , IFN- γ , IL-12 and IL-18 cytokine secretion and/or IgG2 antibody production.
- 5 b. Any change in the number of lymphocytes, levels of secreted IFN β , IFN- α , IFN- γ , IL-12 and IL-18 cytokines, IgG1 or IgG2 antibody levels or IgE antibody levels in the cell culture after contact with the ISS-ODN is measured.
- c. The cells are contacted with the candidate IIS-ON.
- 10 d. Any change in the number of lymphocytes, levels of secreted cytokines, IgG2 antibody levels or IgE antibody levels in the population of cells after contact with the oligonucleotide is measured.

A decline in any of these values (except IgG1 and IgE antibodies) as compared to the measurements taken in step (2) indicates that the candidate oligonucleotide is an IIS-ON
15 of the invention; i.e., it inhibits the immunostimulatory activity of ISS-ODN.

Alternatively, a rise in measured levels of IgG1 or IgE antibodies is an indirect indicator of a rise in a Th2-type lymphocyte response, indicating that the Th1 stimulatory activity of ISS-ODN has declined in the presence of the IIS-ODN. Assay techniques suitable for use in performing the steps above are illustrated in the Examples below. In view of the
20 teaching of this disclosure, other assay techniques for measuring changes in ISS-ODN induced lymphocyte proliferation or cytokine secretion will be apparent to those of ordinary skill in the art.

The screening method can also be used to detect ISS-ODN in a sample of immune cells taken from the host. This aspect of the invention is useful in confirming the presence of

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ISS-ODN containing antigens (e.g., microbial antigens) and autoantigens in the host. To this end, the steps of the above-described screening method are modified to include the steps of:

- 5 a. Obtaining a sample of immune cells from the host, which cells are believed to been exposed to an antigen or autoantigen.
- b. Measuring the levels of lymphocyte proliferation in; IFN β , IFN- α , IFN- γ , IL-12 and IL-18 cytokine secretion from; IgG1 and IgG2 antibody production by; or IgE antibody production by, the sample of host immune cells.
- 10 c. Contacting the sample of host immune cells with an IIS-ON.
- d. Measuring any change in the number of lymphocytes or levels of secreted IFN β , IFN- α , IFN- γ , IL-12 and IL-18 cytokines and/or levels of IgE or IgG1 antibodies in the sample of host immune cells after contact with the IIS-ON, wherein a decline in any of the
15 measured values for lymphocyte proliferation, cytokine secretion or IgG2 antibody production, as well as an increase in IgG1 and IgE antibody production, as compared to the measurements taken in step (b) indicates that an ISS-ODN subject to inhibition by the IIS-ON is present in the sample of host immune cells.

20 2. *Exemplary IIS-ON Structure*

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Particular IIS-ON which inhibit the activity of CpG motif-containing ISS-ODN include those oligonucleotides which are comprised of the following general primary structure:

5'-Purine-Purine-[Y]-[Z]-Pyrimidine-Pyrimidine-3' or

5'-Purine-Purine-[Y]-[Z]-Pyrimidine-polyPyrimidine-3'

- 5 where Y is any naturally occurring or synthetic nucleotide except cytosine and is preferably guanosine, adenosine or inosine (for RNA IIS-ON), most preferably guanosine. In general, Z is any naturally occurring or synthetic nucleotide or repeat of the same nucleotide. Preferably, where Y is inosine, Z is inosine or one or more guanosine(s). Where Y is guanosine, Z is preferably guanosine or one or more
- 10 unmethylated cytosine(s). Where Y is adenosine, Z is preferably guanosine. However, when Y is not guanosine, adenosine or inosine, Z is guanosine, adenosine or inosine. Most preferably, the 5' purines are the same nucleotide, as are the 3' pyrimidines. For example, where ** is YZ, the 5' purines and 3' pyrimidines may be AA**TT, AG**TT, GA**TT, GG**TT, AA**TC, AG**TC, and so forth.
- 15 The core hexamer structure of the foregoing IIS-ON may be flanked upstream and/or downstream by any number or composition of nucleotides or nucleosides. However, IIS-ON will preferably be either 6 mer in length, or between 6 and 45 mer in length, to enhance uptake of the IIS-ON and to minimize non-specific interactions between the IIS-ON and the target recombinant expression vector or host cells. Preferably, any IIS-ON
- 20 flanking sequences present are constructed to match the flanking sequences present in any known ISS-ODN (such as the flanking sequence DY1038 (TTGACTGTG*****AGAGATGA), where ***** is the immunostimulatory hexamer sequence. Those of ordinary skill in the art will be familiar with, or can readily identify, reported nucleotide sequences of known ISS-ODN. For ease of reference in this regard,
- 25 the following sources are especially helpful:

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Yamamoto, *et al.*, *Microbiol. Immunol.*, 36:983 (1992)

Ballas, *et al.*, *J. Immunol.*, 157:1840 (1996)

Klinman, *et al.*, *J. Immunol.*, 158:3635 (1997)

Sato, *et al.*, *Science*, 273:352 (1996)

- 5 Each of these articles are incorporated herein by reference for the purpose of illustrating the level of knowledge in the art concerning the nucleotide composition of ISS-ODN.

Particular inhibitory IIS-ON of the invention include those having the following hexamer sequences:

- 10 1. IIS-ODN having "GG" dinucleotides: AAGGTT, AGGGTT, GGGGTT, GGGGTC, AAGGTC, AAGGCC, AGGGTT, AGGGTC, GAGGTT, GAGGTC, GAGGCC, GGGGCT and so forth.
2. IIS-ODN having "GC" dinucleotides: AAGCTT, AGGCTC, AGGCCC, GAGCTT, GAGCTC, GAGCCC, GGGCTT, GGGCTC, GGGCCC, AAGCCC, AAGCCT, AGGCCT, GAGCCT and so forth.
- 15 3. Inosine and/or adenosine substitutions for nucleotides in the foregoing hexamer sequences made according to the formulae set forth above.

IIS-ON hexamers with especially strong expected inhibitory activity are those with GG and GC competing dinucleotides, particularly AAGGTT (DY1019 in the Figures), AAGCTT (DY1041 in the Figures), AGGGCT, and GAGGTT (including their 3' 20 Pyrimidine-pPyrimidine analogs).

IIS-ON may be single-stranded or double-stranded DNA, single or double-stranded RNA and/or oligonucleosides. The nucleotide bases of the IIS-ON which flank the competing dinucleotides may be the known naturally occurring bases or synthetic non-natural bases

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(e.g., TCAG or, in RNA, UACGI). Oligonucleosides may be incorporated into the internal region and/or termini of the IIS-ON using conventional techniques for use as attachment points for other compounds (e.g., peptides). The base(s), sugar moiety, phosphate groups and termini of the IIS-ON may also be modified in any manner known
5 to those of ordinary skill in the art to construct an IIS-ON having properties desired in addition to the inhibitory activity of the IIS-ON. For example, sugar moieties may be attached to nucleotide bases of IIS-ON in any steric configuration. In addition, backbone phosphate group modifications (e.g., methylphosphonate, phosphorothioate, phosphoroamidate and phosphorodithioate internucleotide linkages) can confer anti-
10 microbial activity on the IIS-ON, making them particularly useful in therapeutic applications.

The techniques for making these phosphate group modifications to oligonucleotides are known in the art and do not require detailed explanation. For review of one such useful technique, the an intermediate phosphate triester for the target oligonucleotide product
15 is prepared and oxidized to the naturally occurring phosphate triester with aqueous iodine or with other agents, such as anhydrous amines. The resulting oligonucleotide phosphoramidates can be treated with sulfur to yield phosphorothioates. The same general technique (excepting the sulfur treatment step) can be applied to yield methylphosphoamidites from methylphosphonates. For more details concerning
20 phosphate group modification techniques, those of ordinary skill in the art may wish to consult U.S. Patent Nos. 4,425,732; 4,458,066; 5,218,103 and 5,453,496, as well as *Tetrahedron Lett.* at 21:4149 (1995), 7:5575 (1986), 25:1437 (1984) and *Journal Am.Chem.Soc.*, 93:6657 (1987), the disclosures of which are incorporated herein for the sole purpose of illustrating the standard level of knowledge in the art concerning
25 preparation of these compounds.

A particularly useful phosphate group modification is the conversion to the phosphorothioate or phosphorodithioate forms of the IIS-ON oligonucleotides. In addition to their potentially anti-microbial properties, phosphorothioates and

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phosphorodithioates are more resistant to degradation *in vivo* than their unmodified oligonucleotide counterparts, making the IIS-ON of the invention more available to the host.

IIS-ON can be synthesized using techniques and nucleic acid synthesis equipment which are well-known in the art. For reference in this regard, see, e.g., Ausubel, *et al.*, *Current Protocols in Molecular Biology*, Chs. 2 and 4 (Wiley Interscience, 1989); Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., New York, 1982); U.S. Patent No. 4,458,066 and U.S. Patent No. 4,650,675. These references are incorporated herein by reference for the sole purpose of demonstrating knowledge in the art concerning production of synthetic oligonucleotides.

Alternatively, IIS-ON can be obtained by mutation of isolated microbial ISS-ODN to substitute a competing dinucleotide for the naturally occurring CpG motif. Screening procedures which rely on nucleic acid hybridization make it possible to isolate any polynucleotide sequence from any organism, provided the appropriate probe or antibody is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligo- peptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can also be deduced from the genetic code, however, the degeneracy of the code must be taken into account.

For example, a cDNA library believed to contain an ISS-containing polynucleotide of interest can be screened by injecting various mRNA derived from cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the desired cDNA expression product, for example, by using antibody specific for a peptide encoded by the polynucleotide of interest or by using probes for the repeat motifs and a tissue expression pattern characteristic of a peptide encoded by the polynucleotide of interest. Alternatively, a cDNA library can be screened indirectly for expression of peptides of interest having at least one epitope using antibodies specific for

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the peptides. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of cDNA of interest.

Once the ISS-containing polynucleotide has been obtained, it can be shortened to the desired length by, for example, enzymatic digestion using conventional techniques. The
5 CpG motif in the ISS-ODN oligonucleotide product is then mutated to substitute a competing dinucleotide for the CpG motif. Techniques for making substitution mutations at particular sites in DNA having a known sequence are well known, for example M13 primer mutagenesis through PCR. Because the IIS-ON is non-coding, there is no concern about maintaining an open reading frame in making the substitution mutation. However,
10 for *in vivo* use, the polynucleotide starting material, ISS-ODN oligonucleotide intermediate or IIS-ON mutation product should be rendered substantially pure (i.e., as free of naturally occurring contaminants and LPS as is possible using available techniques known to and chosen by one of ordinary skill in the art).

The IIS-ON of the invention may be used alone or may be incorporated in *cis* or in *trans*
15 into a recombinant expression vector (plasmid, cosmid, virus or retrovirus) which may in turn code for any therapeutically beneficial protein deliverable by a recombinant expression vector. For the sake of convenience, the IIS-ON are preferably administered without incorporation into an expression vector. However, if incorporation into an expression vector is desired, such incorporation may be accomplished using conventional
20 techniques which do not require detailed explanation to one of ordinary skill in the art. For review, however, those of ordinary skill may wish to consult Ausubel, *Current Protocols in Molecular Biology*, supra.

Briefly, construction of recombinant expression vectors employs standard ligation techniques. For analysis to confirm correct sequences in vectors constructed, the ligation
25 mixtures may be used to transform a host cell and successful transformants selected by antibiotic resistance where appropriate. Vectors from the transformants are prepared, analyzed by restriction and/or sequenced by, for example, the method of Messing, *et al.*,

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(*Nucleic Acids Res.*, 9:309, 1981), the method of Maxam, *et al.*, (*Methods in Enzymology*, 65:499, 1980), or other suitable methods which will be known to those skilled in the art. Size separation of cleaved fragments is performed using conventional gel electrophoresis as described, for example, by Maniatis, *et al.*, (*Molecular Cloning*, pp. 5 133-134, 1982).

Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, 10 and will be apparent to the ordinarily skilled artisan.

If a recombinant expression vector is utilized as a carrier for the IIS-ON of the invention, plasmids and cosmids are particularly preferred for their lack of pathogenicity. However, plasmids and cosmids are subject to degradation *in vivo* more quickly than viruses and therefore may not deliver an adequate dosage of IIS-ON to substantially inhibit ISS-ODN 15 immunostimulatory activity exerted by a systemically administered gene therapy vector. Of the viral vector alternatives, adeno-associated viruses would possess the advantage of low pathogenicity. The relatively low capacity of adeno-associated viruses for insertion of foreign genes would pose no problem in this context due to the relatively small size in which IIS-ON of the invention can be synthesized.

20 Other viral vectors that can be utilized in the invention include adenovirus, adeno-associated virus, herpes virus, vaccinia or an RNA virus such as a retrovirus. Retroviral vectors are preferably derivatives of a murine, avian or human HIV retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus 25 (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these

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vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper
5 cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence that enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines that have deletions of the packaging signal include, but are not limited to, Ψ2, PA317 and PA12, for example. These cell lines
10 produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such helper cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion can be produced.

By inserting one or more sequences of interest into the viral vector, along with another
15 gene which encodes the ligand for a receptor on a specific target cell, for example, the vector can be rendered target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue
20 experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing the polynucleotides of interest.

Alternatively, a colloidal dispersion system may be used for targeted delivery. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres,
25 beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome.

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- Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated
- 5 within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al.*, *Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes encoding the antisense polynucleotides
- 10 at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.*, *Biotechniques*, 6:682, 1988).
- 15 The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.
- 20 Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include
- 25 egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

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The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency
5 of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the
10 naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various well known linking groups can be used for joining
15 the lipid chains to the targeting ligand (see, e.g., Yanagawa, *et al.*, *Nuc.Acids Symp.Ser.*, 19:189 (1988); Grabarek, *et al.*, *Anal.Biochem.*, 185:131 (1990); Staros, *et al.*, *Anal.Biochem.*, 156:220 (1986) and Boujrad, *et al.*, *Proc.Natl.Acad.Sci.USA*, 90:5728 (1993), the disclosures of which are incorporated herein by reference solely to illustrate the standard level of knowledge in the art concerning conjugation of oligonucleotides to
20 lipids).

Targeted delivery of IIS-ON can also be achieved by conjugation of the IIS-ON to a the surface of viral and non-viral recombinant expression vectors, to an antigen or other ligand, to a monoclonal antibody or to any molecule which has the desired binding specificity. A particular IIS-ODN conjugate of interest is one in which an autoantigen
25 or autoantibody is the IIS-ODN conjugate partner. IIS-ODN autoantigen conjugates are useful in boosting host Th2 type immune responses to the autoantigen (suppressing the Th1 responses induced by the autoantigen itself; see, e.g., Conboy, *et al.*, *J.Exp.Med.*, 185:439-451 (1997)), while IIS-ODN autoantibody conjugates are useful in inducing

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passive immunity in a host suffering from an autoimmune condition. Specific methods for delivery of such conjugates, as well as IIS-ON in general, are described in greater detail *infra*.

- Those of ordinary skill in the art will be familiar with, or can readily determine, sources for autoantigens and autoantibodies useful as IIS-ON conjugates. Examples of such conjugate materials include myelin basic protein (*see, e.g.,* sequence and sourcing information provided in Segal, *et al., J.Immunol.*, 158:5087 (1997); Matsuo, *et al., Am.J.Pathol.*, 150:1253 (1997); and Schluesener, *FEMS Immunol.Med.Microbiol.*, 17:179 (1997)); Sjorgen's syndrome autoantigen (*see, e.g.,* Hanjei, *et al., Science*, 276:604 (1997)); hemochromatosis autoantigen (*see, e.g.,* Ruddy, *et al., Genome Res.*, 7:441 (1997)), La/SSB protein (*see, e.g.,* Castro, *et al., Cell Calcium*, 20:493 (1996)); HsEg5 lupus autoantigen (*see, e.g.,* Whitehead, *et al., Arthritis Rheum.*, 39:1635 (1996)); Ki nuclear lupus autoantigen (*see, e.g.,* Paesen and Nuttal, *Biochem.Biophys.Acta*, 1309:9 (1996)); and antibodies thereto (*see, e.g.,* Menon, *et al., J.Autoimmun.*, 10:43 (1997) and Rahman, *et al., Semin.Arthritis Rheum.*, 26:515 (1996) [human antiphospholipid (anti-DNA) monoclonal antibodies]; and, Kramers, *et al., J.Autoimmun.*, 9:723 (1997) [monoclonal anti-nucleosome lupus autoantibodies]). Each of the cited references is incorporated herein solely to illustrate the level of knowledge and skill in the art concerning the identity, activity and structure of autoantigens and autoantibodies.
- Examples of other useful conjugate partners include any immunogenic antigen (including allergens, live and attenuated viral particles and tumor antigens), targeting peptides (such as receptor ligands, antibodies and antibody fragments, hormones and enzymes), non-peptidic antigens (coupled via a peptide linkage, such as lipids, polysaccharides, glycoproteins, gangliosides and the like) and cytokines (including interleukins, interferons, erythropoietin, tumor necrosis factor and colony stimulating factors). Such conjugate partners can be prepared according to conventional techniques (*e.g.,* peptide synthesis) and many are commercially available.

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Those of ordinary skill in the art will also be familiar with, or can readily determine, methods useful in preparing oligonucleotide-peptide conjugates. Conjugation can be accomplished at either termini of the IIS-ON or at a suitably modified base in an internal position (e.g., a cytosine or uracil). For reference, methods for conjugating
5 oligonucleotides to proteins and to oligosaccharide moieties of Ig are known (see, e.g., O'Shannessy, *et al.*, *J. Applied Biochem.*, 7:347 (1985), the disclosure of which is incorporated herein by reference solely to illustrate the standard level of knowledge in the art concerning oligonucleotide conjugation). Another useful reference is Kessler: "Nonradioactive Labeling Methods for Nucleic Acids", in Kricka (ed.), *Nonisotopic DNA*
10 *Probe Techniques* (Acad. Press, 1992)).

Briefly, examples of known, suitable conjugation methods include: conjugation through 3' attachment via solid support chemistry (see, e.g., Haralambidis, *et al.*, *Nuc. Acids Res.*, 18:493 (1990) and Haralambidis, *et al.*, *Nuc. Acids Res.*, 18:501 (1990) [solid support synthesis of peptide partner]; Zuckermann, *et al.*, *Nuc. Acids Res.*, 15:5305 (1987), Corey,
15 *et al.*, *Science*, 238:1401 (1987) and Nelson, *et al.*, *Nuc. Acids Res.*, 17:1781 (1989) [solid support synthesis of oligonucleotide partner]). Amino-amino group linkages may be performed as described in Benoit, *et al.*, *Neuromethods*, 6:43 (1987), while thiol-carboxyl group linkages may be performed as described in Sinah, *et al.*, *Oligonucleotide Analogues: A Practical Approach* (IRL Press, 1991). In these latter methods, the
20 oligonucleotide partner is synthesized on a solid support and a linking group comprising a protected amine, thiol or carboxyl group opposite a phosphoramidite is covalently attached to the 5'-hydroxyl (see, e.g., U.S. Patent Nos. 4,849,513; 5,015,733; 5,118,800 and 5,118,802).

Linkage of the oligonucleotide partner to a peptide may also be made via incorporation
25 of a linker arm (e.g., amine or carboxyl group) to a modified cytosine or uracil base (see, e.g., Ruth, *4th Annual Congress for Recombinant DNA Research* at 123). Affinity linkages (e.g., biotin-streptavidin) may also be used (see, e.g., Roget, *et al.*, *Nuc. Acids Res.*, 17:7643 (1989)).

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Methods for linking oligonucleotides to lipids are also known and include synthesis of oligo-phospholipid conjugates (see, e.g., Yanagawa, *et al.*, *Nuc. Acids Symp.Ser.*, 19:189 (1988)), synthesis of oligo-fatty acids conjugates (see, e.g., Grabarek, *et al.*, *Anal.Biochem.*, 185:131 (1990)) and oligo-sterol conjugates (see, e.g., Boujrad, *et al.*,
5 *Proc.Natl.Acad.Sci USA*, 90:5728 (1993)).

Each of the foregoing references is incorporated herein by reference for the sole purpose of illustrating the level of knowledge and skill in the art with respect to oligonucleotide conjugation methods.

If to be delivered without use of a vector or other delivery system, IIS-ON will be
10 prepared in a pharmaceutically acceptable composition. Pharmaceutically acceptable carriers preferred for use with the IIS-ON of the invention may include sterile aqueous of non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/
15 aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example,
20 antimicrobials, antioxidants, chelating agents, and inert gases and the like. A composition of IIS-ON may also be lyophilized using means well known in the art, for subsequent reconstitution and use according to the invention.

B. Methods for Administering and Using IIS-ON of the Invention

The IIS-ON of the invention are useful in inhibiting the immunostimulatory activity of
25 ISS, wherever present. Thus, IIS-ON are useful as, for example, anti-inflammatory agents for reducing host immune responses to ISS-ODN in bacteria and viruses. IIS-ON

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are also useful as agents for suppressing the immunostimulatory activity of any ISS-ODN, known or unknown, present in recombinant expression vectors, especially those used for gene therapy and immunization. In addition, IIS-ODN are useful in inhibiting host autoimmune responses stimulated by microbial ISS-ODN and in boosting Th2 type
5 responses to antigen.

In this context, "inhibition" refers to a reduction in the host immune response as compared to the level of ISS-ODN stimulated host immune response prior to IIS-ODN administration. Because ISS-ODN stimulate secretion of certain cytokines (e.g., IL-12, IL-18 and IFNs) and tend to shift the host cellular immune response to the Th1
10 repertoire, measurements of cytokine levels, cytokine-stimulated lymphocyte proliferation, IgG2 antibody levels (the production of which is indicative of a Th1 lymphocyte response), IgE levels (the suppression of which is indicative of a Th1 lymphocyte response) and IgG1 antibody levels (the production of which is indicative of a Th2 lymphocyte response) are all suitable values for use in detecting IIS-ODN inhibitory
15 activity. Specific examples and details of methods for determining such values are described further *infra*.

With respect to shifts in the Th1/Th2 repertoire and consequent changes in cytokine levels, it is helpful to recall that CD4+ lymphocytes generally fall into one of two distinct subsets; i.e., the Th1 and Th2 cells. Th1 cells principally secrete IL-2, IFN γ and
20 TNF β (the latter two of which mediate macrophage activation and delayed type hypersensitivity) while Th2 cells principally secrete IL-4 (which stimulates production of IgE antibodies), IL-5, IL-6 and IL-10. These CD4+ subsets exert a negative influence on one another; i.e., secretion of Th1 lymphokines inhibits secretion of Th2 lymphokines and vice versa. In addition, it is believed that exposure of Th2 cells to cytotoxic T
25 lymphocytes (CTLs) also suppresses TH2 cell activity.

Factors believed to favor Th1 activation resemble those induced by viral infection and include intracellular pathogens, exposure to IFN- β , IFN- α , IFN γ , IL-12 and IL-18, as

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- well as the presence of APCs and exposure to low doses of antigen. Th1 type immune responses also predominate in autoimmune disease. Factors believed to favor Th2 activation include exposure to IL-4 and IL-10, APC activity on the part of B lymphocytes and high doses of antigen. Active Th1 (IFN γ) cells enhance cellular immunity and are therefore of particular value in responding to intracellular infections, while active Th2 cells enhance antibody production and are therefore of value in responding to extracellular infections (albeit at the risk of anaphylactic events associated with IL-4 stimulated induction of IgE antibody production). Thus, the ability to shift host immune responses from the Th1 to the Th2 repertoire and vice versa has substantial clinical significance for enhancing and controlling host immunity against infection and allergy. Further, control over Th1/Th2 mediated cytokine release enables one to control host immune responses to self-antigens (having clinical significance for treatment of autoimmune disease) and to recombinant expression vector antigens (having clinical significance for control of gene expression for gene therapy and gene immunization).
- 15 For use in modulating the immunogenicity of a recombinant expression vector, the IIS-ON of the invention will be administered according to any means and route by which the target recombinant expression vector is administered to a host, including *in vivo* and *ex vivo* routes. Uptake of IIS-ON by host cells occurs at least as robustly as does uptake of therapy and immunization vectors, if not more so due to the small size of IIS-ON as compared to the total dimensions of plasmid, viral and retroviral nucleic acids.

- A particular goal of IIS-ON administration in this context is the inhibition of ISS-ODN stimulated, Th1 mediated cytokine production. Thus, a measurable reduction of such cytokine levels in a treated host constitutes IIS-ON therapeutic activity in this embodiment of the invention. IIS-ON therapeutic activity is also demonstrated in this context by prolongation of gene expression as compared to expression levels obtained in the absence of IIS-ON. Those of ordinary skill in the gene therapy and immunization arts will be very familiar with, or can readily ascertain, clinically acceptable means and routes for administration of therapy and immunization vectors and, by extension, IIS-ON.

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For use as anti-inflammatory agents, IIS-ON and IIS-ON conjugates will be administered according to any means and route by which known anti-inflammatories and antibiotics are administered. A particular goal of IIS-ODN administration in this context is the inhibition of ISS-ODN stimulated, Th1 mediated cytokine production. Thus, a
5 measurable reduction of such cytokine levels in a treated host constitutes IIS-ODN therapeutic activity in this embodiment of the invention. Those of ordinary skill in the art of treating infectious disease will be very familiar with, or can readily ascertain, clinically acceptable means and routes for administration of anti-inflammatories and antibiotics and, by extension, IIS-ON and their conjugates.

10 For use as autoimmune modulators, IIS-ON and IIS-ON autoantigen or autoantibody conjugates will be administered according to any means and route by which known therapies for autoimmune disease are practiced. A particular goal of IIS-ODN administration in this context is the inhibition of ISS-ODN stimulated, Th1 mediated IL-12 production. Thus, a measurable reduction of IL-12 levels in an autoimmune host
15 constitutes IIS-ODN therapeutic activity in this embodiment of the invention. Those of ordinary skill in the art of treating autoimmune disease will be very familiar with, or can readily ascertain, clinically acceptable means and routes for administration of IIS-ON and their conjugates.

For use as modulators of ISS-ODN administered as immunostimulants, the IIS-ON and
20 IIS-ON conjugates of the invention will be administered according to any means and route by which the target ISS-ODN is administered to a host, including *in vivo* and *ex vivo* routes. For example, where ISS-ODN are administered as adjuvants in an immunization protocol (see, co-pending and commonly assigned U.S. Patent Applications Serial Nos. 60/028,118 and 08/593,554), it may be desirable to be able to
25 subsequently reduce or eliminate the ISS-ODN immunostimulatory activity to modify the course of therapy. In this context, therefore, IIS-ON serve as ISS-ODN "off" switches, whereby IIS-ON and IIS-ON conjugate activity is demonstrated by a measured

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reduction in ISS-ODN stimulated cytokine production, ISS-ODN stimulated lymphocyte production, or a shift away from the Th1 lymphocyte repertoire.

For use as adjuvants for Th2 immune responses to extracellular antigen, the IIS-ON of the invention will be administered according to any means and route by which antigen-based vaccines may be administered to a host. Shifts away from the Th1 lymphocyte repertoire are a measure of efficacy for use of IIS-ON and IIS-ON conjugates as Th2 lymphocyte stimulatory adjuvants in the presence of antigen.

A particular advantage of the IIS-ON of the invention is their capacity to exert an ISS-ODN inhibitory activity even at relatively low dosages. Although the dosage used will vary depending on the clinical goals to be achieved, a suitable dosage range is one which provides up to about 1-200 μg of IIS-ON/ml of carrier in a single dosage. In view of the teaching provided by this disclosure, those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, suitable parameters for administration of IIS-ON according to the invention.

15 In this respect, the inhibitory activity of IIS-ON is essentially dose-dependent. Therefore, to increase IIS-ON potency by a magnitude of two, each single dose is doubled in concentration. For use in inhibiting ISS-ODN activity (including activity of ISS-ODN in recombinant expression vectors), it is useful to administer the IIS-ON and target ISS-ODN or vector in equivalent dosages, then increase the dosage of IIS-ON as
20 needed to achieve the desired level of inhibition. For use as an anti-inflammatory agent, it is useful to administer the IIS-ON in a low dosage (e.g., about 1 $\mu\text{g}/\text{ml}$ to about 50 $\mu\text{g}/\text{ml}$), then increase the dosage as needed to achieve the desired therapeutic goal. Alternatively, a target dosage of IIS-ON can be considered to be about 1-10 μM in a sample of host blood drawn within the first 24-48 hours after administration of IIS-ON.

25 To maximize the effectiveness of IIS-ON to inhibit ISS-ODN immunostimulatory activity, the IIS-ON are preferably co-administered with the target ISS-ODN or

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recombinant expression vector. In addition, IIS-ON may be pre-incubated with the target recombinant expression vector prior to administration to the host to reduce the latter's capacity to present ISS-ODN immunostimulatory activity in the host during treatment in a therapy or immunization regime. For use as an anti-inflammatory, the IIS-ON may
5 be co-administered with, or otherwise taken by a host treated with, other anti-inflammatory pharmaceuticals.

To these ends, IIS-ON are conveniently supplied in single dose vials and/or in kits together with suitable dosages of ISS-ODN, recombinant expression vectors or anti-inflammatory agents. In kits including recombinant expression vectors, the IIS-ON and
10 vectors can be pre-mixed in single dosage vials. Means for administering each dosage to a host (e.g., syringes, transdermal patches, iontophoresis devices and inhalers), if required, are included in each kit.

Examples illustrating the immunoinhibitory activity of IIS-ON are set forth below. The examples are for purposes of reference only and should not be construed to limit the
15 invention, which is to be defined by the appended claims. All abbreviations and terms used in the examples have their expected and ordinary meaning unless otherwise specified.

EXAMPLE I

ASSAY TO CONFIRM IIS-ON INHIBITORY ACTIVITY

20 AS MEASURED BY A REDUCTION IN LYMPHOCYTE PROLIFERATION

Splenocytes from immunologically naive female Balb/c mice (6-8 weeks of age) were harvested from each animal. Supernatants of the harvested splenocytes were incubated with 1 µg/ml of the DY1018 ISS-ODN or 1 µg/ml of the DY1038 ISS-ODN in normal saline (all oligonucleotide sequences are set forth in the legend to the FIGURES and in
25 the Description of Drawings). The backbones of both DY1018 and DY1038 were

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modified as phosphorothioates. In this context, the ISS-ODN served as non-specific adjuvants for *in vitro* stimulation of the immune system.

- Within 4 hours of ISS-ODN contact, the supernatants were incubated with various concentrations of IIS-ON or a control. DY1039 (an ISS with the cytosine methylated),
- 5 DY1040 and DY1043 (the latter with CC dinucleotides in place of the CG dinucleotide of DY1018 and DY1038) served as controls. To confirm the location of potential competition with DY1018 and DY1038, all of the oligonucleotides were identical to DY1038 (FIGURES 1 and 3) or DY1018 (FIGURE 2) except for the hexamer regions identified in the FIGURES and DY1043 (an irrelevant sequence control).
- 10 Lymphocyte proliferation pre- and post-IIS-ODN administration was measured (as a function of counts per minute) using conventional assay techniques. Any observable changes in lymphocyte proliferation among the supernatants were noted. Values shown in FIGURES 1 through 3 are averages for each group of mice tested.

- The results of these assays are shown in FIGURES 1 through 3. With respect to both
- 15 DY1038 (FIGURES 1 and 3) and DY1018 (FIGURE 2), the strongest inhibition of ISS immunostimulatory activity by inhibitory IIS-ON of the invention in these experiments was demonstrated by IIS-ON DY1019 (having a hexamer region consisting of AAGGTT). Other strongly inhibitory IIS-ON tested were DY1048 (hexamer region=GAGGTC), DY1050 (hexamer region=AGGGCT), DY1060 (hexamer re-
- 20 gion=TTGCAA) and DY1041 (hexamer region=AAGCTT) (FIGURE 3). Inhibitory strength was dose-dependent in a generally proportional relationship of dosage to reduction in lymphocyte proliferation measured.

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EXAMPLE II

ASSAY TO CONFIRM IIS-ON INHIBITORY ACTIVITY AS MEASURED BY A REDUCTION IN INF- γ SECRETION

Groups of mice were immunized as described in Example I, sacrificed and their
5 splenocytes harvested. Supernatants of harvested splenocytes was incubated with 1
 μ g/ml of DY1018 ISS-ODN in saline as described in Example I. Within 4 hours, the
supernatants were incubated with various concentrations of IIS-ON or a control.
DY1039 (an ISS with the cytosine methylated), DY1040 and DY1043 (the latter with CC
dinucleotides in place of the CG dinucleotide of DY1018) served as controls (all
10 oligonucleotide sequences are set forth in the legend to the FIGURES and in the
Description of Drawings). To confirm the location of potential competition with
DY1018, all of the oligonucleotides were identical to DY1018 except for the hexamer
regions identified and DY1043 (an irrelevant sequence control).

IFN- γ levels were measured pre- and post- IIS-ODN contact. Any observable changes
15 in IFN- γ secretion (pg/ml supernatants) among the supernatants were noted. Values
shown in FIGURE 4 are averages for each group of mice tested.

The results of these assays are shown in FIGURE 4. Again, the strongest inhibition of
ISS immunostimulatory activity by inhibitory IIS-ON of the invention in these
experiments was demonstrated by IIS-ON DY1019 (having a hexamer region consisting
20 of AAGGTT). DY1041 (hexamer region=AAGCTT) was also strongly inhibitory, even
at low dosage (1 μ g/ml saline). At a higher dosage (10 μ g/ml), INF- γ levels began to
decline in control mice as well.

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EXAMPLE III

IIS-ODN BOOSTING OF Th2 TYPE IMMUNE RESPONSES
TO ANTIGEN

- Groups of four Balb/c mice were co-immunized with 10 µg β-galactosidase antigen and 5 50 µg (in 50 µl normal saline) of IIS-ODN DY1019 (identified in the Figure as β-gal/M-ODN), the ISS-ODN composition β-gal/ISS-ODN (5'-AATTCAACGTTTCGC-3'), the β-gal antigen and pKISS-3 (a plasmid having three copies of the AACGTT ISS-ODN hexamer in the backbone), the β-gal antigen and pKISS-0 (a control plasmid having no copies of the AACGTT ISS-ODN hexamer in the backbone), or saline alone. Th2 10 responses in each group of mice were measured by ELISA as a function of IgE levels obtained post-boosting. As shown in FIGURE 5, potent Th2-type responses (above 1000 CPM) were obtained only in the mice which received saline (approximately 1200 CPM at 1 week post-boosting) and β-gal/M-ODN (approximately 1750 CPM at 1 week post-boosting).
- 15 Further, high levels of IgG2a antibodies and low levels of IgG1 antibodies (Th1 and Th2 type responses, respectively) were induced in response to antigen in the ISS-ODN treated mice, while the opposite responses were obtained in the IIS-ON treated mice, thus showing a shift toward the Th2 repertoire in the latter group.

- The invention having been fully described, modifications of the disclosed embodiments 20 may become apparent to those of ordinary skill in the art. All such modifications are considered to be within the scope of the invention, which is defined by the appended claims.

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CLAIMS

The invention claimed is:

1. A pharmaceutically useful compound for inhibiting immunostimulation by immunostimulatory sequence oligodeoxynucleotides (ISS-ODN), wherein the
5 ISS-ODN contain a hexamer region consisting of at least one CpG nucleotide motif flanked by two 5' purines and two 3' pyrimidines (ISS-ODN), comprising:

an oligonucleotide containing a hexamer region having the nucleotide sequence 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-Pyrimidine-3' or 5'-Purine-Purine-[Y]-[Z]-
10 Pyrimidine-poly(Pyrimidine)-3';

where Y is any naturally occurring or synthetic nucleotide except cytosine and Z is any naturally occurring or synthetic nucleotide; however, when Y is not guanosine or inosine, Z is guanosine or inosine.
2. The compound according to Claim 1 where Y is guanosine or inosine.
- 15 3. The compound according to Claim 1 where Y is inosine and Z is inosine or guanosine.
4. The compound according to Claim 1 where Y is guanosine and Z is guanosine or an unmethylated cytosine.
5. A pharmaceutically useful compound for inhibiting immunostimulation by
20 immunostimulatory sequence oligodeoxynucleotides comprising an oligonucleotide containing a hexamer region having the nucleotide sequence AAGGTT.

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6. A pharmaceutically useful compound for inhibiting immunostimulation by immunostimulatory sequence oligodeoxynucleotides comprising an oligonucleotide containing a hexamer region having a nucleotide sequence consisting of AAGCTT.
- 5 7. A pharmaceutically useful compound for inhibiting immunostimulation by immunostimulatory sequence oligodeoxynucleotides comprising an oligonucleotide containing a hexamer region having a nucleotide sequence consisting of AGGGCT
8. A pharmaceutically useful compound for inhibiting immunostimulation by
10 immunostimulatory sequence oligodeoxynucleotides comprising an oligonucleotide containing a hexamer region having a nucleotide sequence consisting of GAGGTT.
9. A pharmaceutically useful compound for inhibiting immunostimulation by
15 immunostimulatory sequence oligodeoxynucleotides comprising an oligonucleotide containing a hexamer region having a nucleotide sequence selected from the group of sequences consisting of AAGCTT, AGGCTC, GAGCTT, GGGCTT, AAGCTC, AGGCTC, GAGCTC, GGGCTC, AAGCCC, AGGCCC, GAGCCC, GGGCCC, AGGCCT, GAGCCT, GGGGCT, TTGCAA, AATGTT, GGGGTT and AAGCCC.
- 20 10. The compound according to any of Claims 1 through 9 wherein the hexamer region is flanked by nucleotides in a sequence identical to the sequence of nucleotides which flank the hexamer region of any known ISS-ODN.
11. The compound according to any of Claims 1 through 9 wherein the oligonucleotide compound is conjugated to a peptide.

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12. A kit for use in gene therapy or gene immunization consisting of any of the immunoinhibitory compounds of Claims 1 through 11 in a sterile vial and a recombinant expression vector in a sterile vial.
13. The kit according to Claim 12 wherein the immunoinhibitory compound and
5 the recombinant expression vector are contained in the same sterile vial.
14. A method for inhibiting the immunostimulatory activity of ISS-ODN in contact with a population of vertebrate cells which includes lymphocytes or monocytes comprising contacting the population of vertebrate cells with an immunoinhibitory amount of an oligonucleotide containing a hexamer region
10 having the nucleotide sequence 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-Pyrimidine-3' or 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-poly(Pyrimidine)-3', where Y is any naturally occurring or synthetic nucleotide except cytosine and Z is any naturally occurring or synthetic nucleotide; however, when Y is not guanosine or inosine, Z is guanosine or inosine; wherein a reduction in
15 Th1 type immune responses measured in the population of vertebrate cells indicates that the desired inhibition of ISS-ODN immunostimulatory activity has been achieved.
15. The method according to Claim 14 wherein the ISS-ODN are believed to be present in a recombinant expression vector.
- 20 16. The method according to Claim 15 wherein both the recombinant expression vector and the immunoinhibitory oligonucleotide are administered to a vertebrate host.
17. The method according to Claim 14 wherein the ISS-ODN are believed to be present in a microbe.

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18. The method according to Claim 17 wherein the microbe has infected a vertebrate host and the microbe is contacted with the immunoinhibitory oligonucleotide by administering the oligonucleotide in an immunoinhibitory amount to the host.
- 5 19. The method according to Claim 18 wherein the vertebrate host has an autoimmune disease believed to be clinically related to infection of the host by the microbe.
20. A method for prolonging gene expression in a recombinant expression vector believed to contain at least one ISS-ODN comprising contacting the
10 recombinant expression vector with an immunoinhibitory amount of an oligonucleotide containing a hexamer region having the nucleotide sequence 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-Pyrimidine-3' or 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-poly(Pyrimidine)-3', where Y is any naturally occurring or
15 synthetic nucleotide except cytosine and Z is any naturally occurring or synthetic nucleotide; however, when Y is not guanosine or inosine, Z is guanosine or inosine; wherein gene expression for a longer period of time than is obtained from the same recombinant expression vector in the absence of contact with the immunoinhibitory oligonucleotide indicates that the desired prolongation of gene expression has been achieved.
- 20 21. The method according to Claim 20 wherein both the recombinant expression vector and the immunoinhibitory oligonucleotide are administered to a vertebrate host.

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22. A method for reducing inflammation in a host in response to a microbial infection of the host comprising administering an immunoinhibitory amount of an oligonucleotide containing a hexamer region having the nucleotide sequence 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-Pyrimidine-3' or 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-poly(Pyrimidine)-3' to the host, where Y is any naturally occurring or synthetic nucleotide except cytosine and Z is any naturally occurring or synthetic nucleotide; however, when Y is not guanosine or inosine, Z is guanosine or inosine; wherein a reduction in Th1 type immune responses against the infectious microbe measured in the host or a reduction in other clinical signs of inflammation in the host indicates that the desired reduction in host inflammation has been achieved.
23. A method for modulating the immunostimulatory activity of an ISS-ODN in contact with a population of vertebrate cells which includes lymphocytes or monocytes comprising contacting the population of vertebrate cells with an immunoinhibitory amount of an oligonucleotide containing a hexamer region having the nucleotide sequence 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-Pyrimidine-3' or 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-poly(Pyrimidine)-3', where Y is any naturally occurring or synthetic nucleotide except cytosine and Z is any naturally occurring or synthetic nucleotide; however, when Y is not guanosine or inosine, Z is guanosine or inosine; wherein a reduction in Th1 type immune responses measured in the population of vertebrate cells indicates that the desired inhibition of ISS-ODN immunostimulatory activity has been achieved.
24. The method according to Claim 23 wherein both the ISS-ODN and the immunoinhibitory oligonucleotide are administered to a vertebrate host.

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25. A method for boosting a Th2 type immune response to an antigen comprising contacting a population of antigen stimulated vertebrate cells including lymphocytes with an immunostimulatory amount of an oligonucleotide containing a hexamer region having the nucleotide sequence 5'-Purine-
5 Purine-[Y]-[Z]-Pyrimidine-Pyrimidine-3' or 5'-Purine-Purine-[Y]-[Z]-
Pyrimidine-poly(Pyrimidine)-3', where Y is any naturally occurring or synthetic nucleotide except cytosine and Z is any naturally occurring or synthetic nucleotide; however, when Y is not guanosine or inosine, Z is guanosine or inosine; wherein wherein a reduction in Th1 type immune
10 responses or increase in antigen stimulated IgG1 production measured in the population of vertebrate cells indicates that the desired boost in Th2 type immune responses to the antigen has been achieved.

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26. A method for identifying IIS-ON which inhibit the immunostimulatory activity of ISS-ODN comprising:
- 5 (a) contacting a population of antigen stimulated immune cells with an ISS-ODN to induce lymphocyte proliferation in; $\text{IFN}\beta$, $\text{IFN}\alpha$, $\text{IFN}\gamma$, IL-12 and IL-18 cytokine secretion from; IgG1 antibody production by; or IgE suppression in, the population of antigen stimulated immune cells;
- (b) measuring any change in the number of lymphocytes or levels of secreted cytokines and/or levels of IgE or IgG1 antibodies in the population of antigen stimulated cells after contact with the ISS-ODN;
- 10 (c) contacting the population of antigen stimulated cells with a candidate IIS-ON inhibitory oligonucleotide; and,
- (d) measuring any change in the number of lymphocytes or levels of secreted $\text{IFN}\beta$, $\text{IFN}\alpha$, $\text{IFN}\gamma$, IL-12 and IL-18 cytokines and/or levels of IgE or IgG1 antibodies in the population of antigen stimulated cells after contact
- 15 with the oligonucleotide, wherein a decline in any of the measured values for lymphocyte proliferation, cytokine secretion or IgG1 antibody production, as well as an increase in IgE antibody production, as compared to the measurements taken in step (b) indicates that the oligonucleotide inhibits the immunostimulatory activity of the ISS-ODN of step (a).
- 20 27. The method according to Claim 26 wherein the candidate inhibitory oligonucleotide contains a hexamer region having the nucleotide sequence 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-Pyrimidine-3' or 5'-Purine-Purine-[Y]-[Z]-Pyrimidine-poly(Pyrimidine)-3', where Y is any naturally occurring or synthetic nucleotide except cytosine and Z is any naturally occurring or

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synthetic nucleotide; however, when Y is not guanosine, adenosine or inosine, Z is guanosine or inosine.

28. A pharmaceutically useful compound comprising an oligonucleotide
5 identified according to the method of Claim 26 as one which inhibits the immunostimulatory activity of ISS-ODN.
29. A method for detecting ISS-ODN immunostimulatory activity in a host comprising:
- 10 (a) obtaining a sample of immune cells from the host, which cells are believed to have been exposed to an antigen or autoantigen;
- (b) measuring the levels of lymphocyte proliferation in; $\text{IFN}\beta$, $\text{IFN}\alpha$, $\text{IFN}\gamma$, IL-12 and IL-18 cytokine secretion from; IgG1 antibody production by; or IgE suppression in, the sample of host immune cells;
- 15 (c) contacting the sample of host immune cells with an immunoinhibitory oligonucleotide (IIS-ON); and,
- (d) measuring any change in the number of lymphocytes or levels of secreted $\text{IFN}\beta$, $\text{IFN}\alpha$, $\text{IFN}\gamma$, IL-12 and IL-18 cytokines and/or levels of IgE, IgG2 or IgG1 antibodies in the sample of host immune cells after contact with the IIS-ON, wherein a decline in any of the measured values for lymphocyte
20 proliferation, cytokine secretion or IgG2 antibody production, as well as an increase in IgG1 or IgE antibody production, as compared to the measurements taken in step (b), indicates that an ISS-ODN subject to inhibition by the IIS-ON is present in the sample of host immune cells.

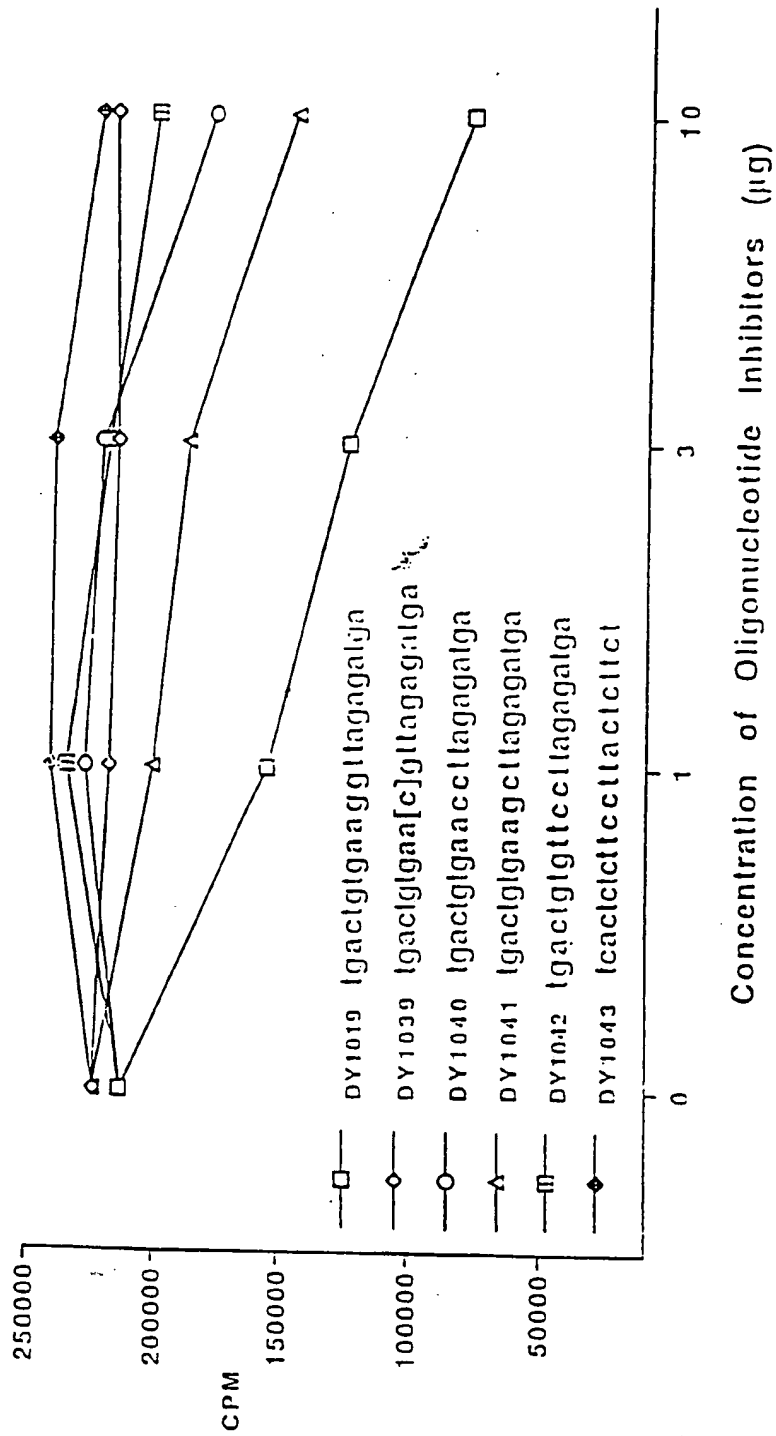


FIGURE 1

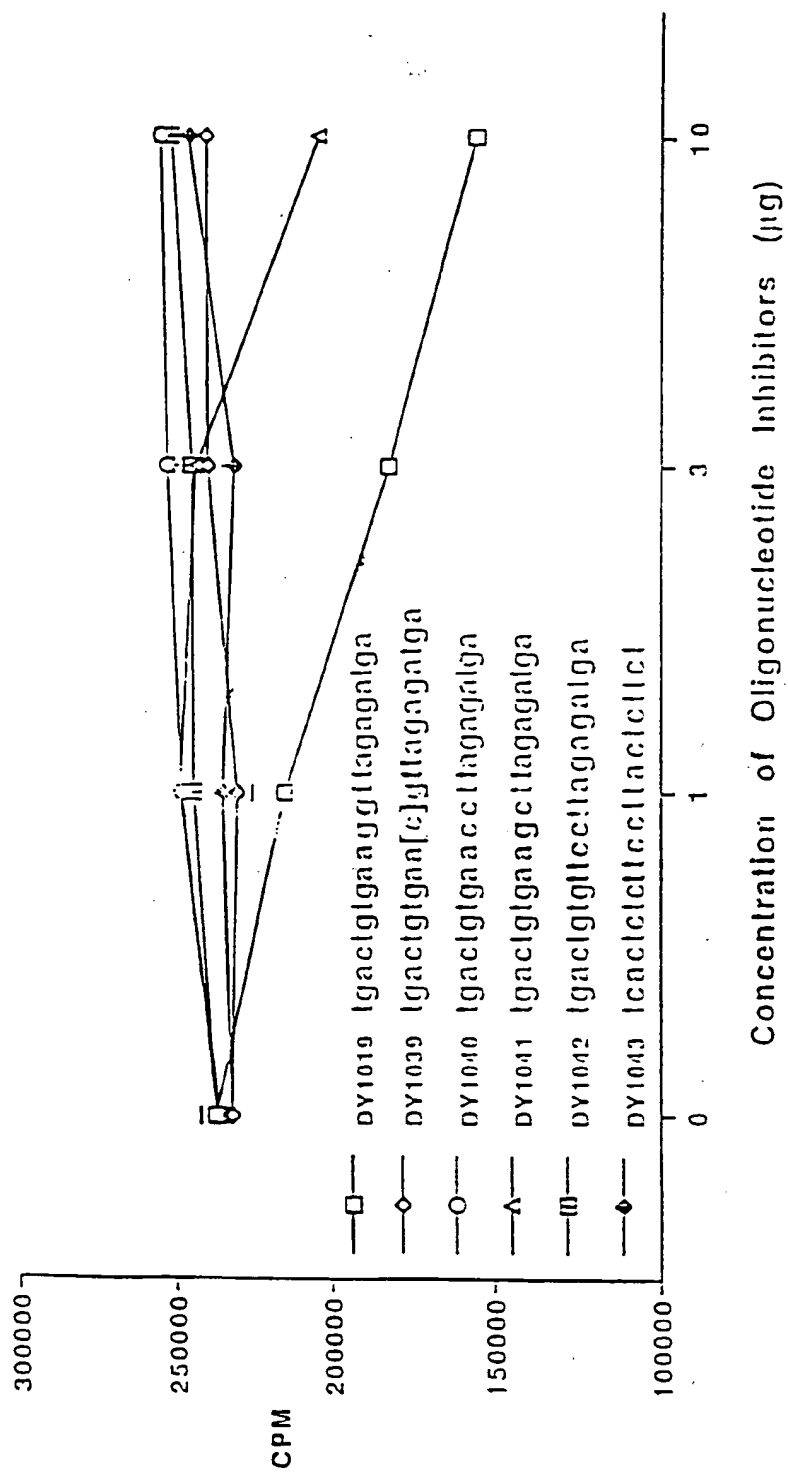


Figure 2

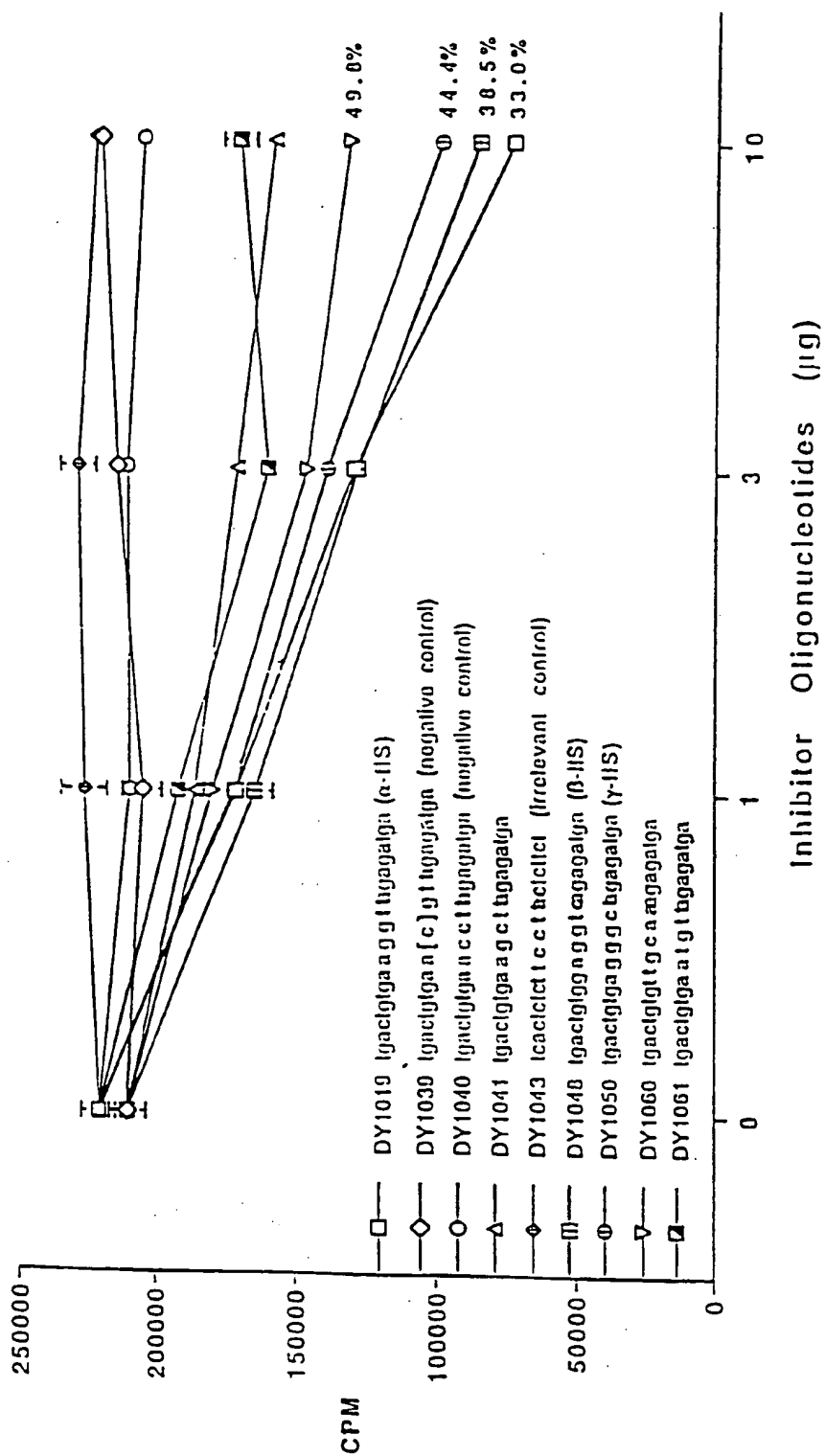


FIGURE 3

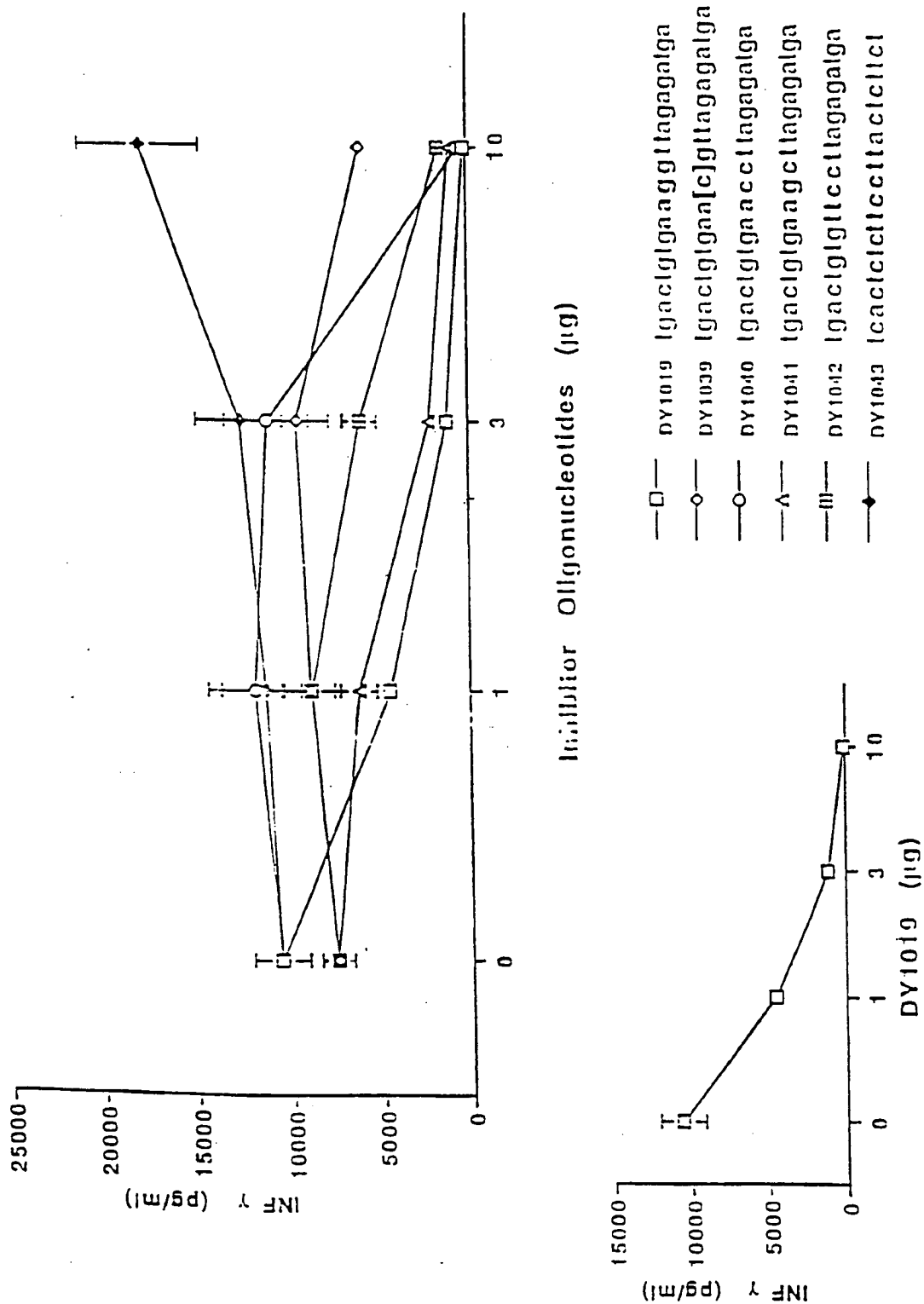


FIGURE 4

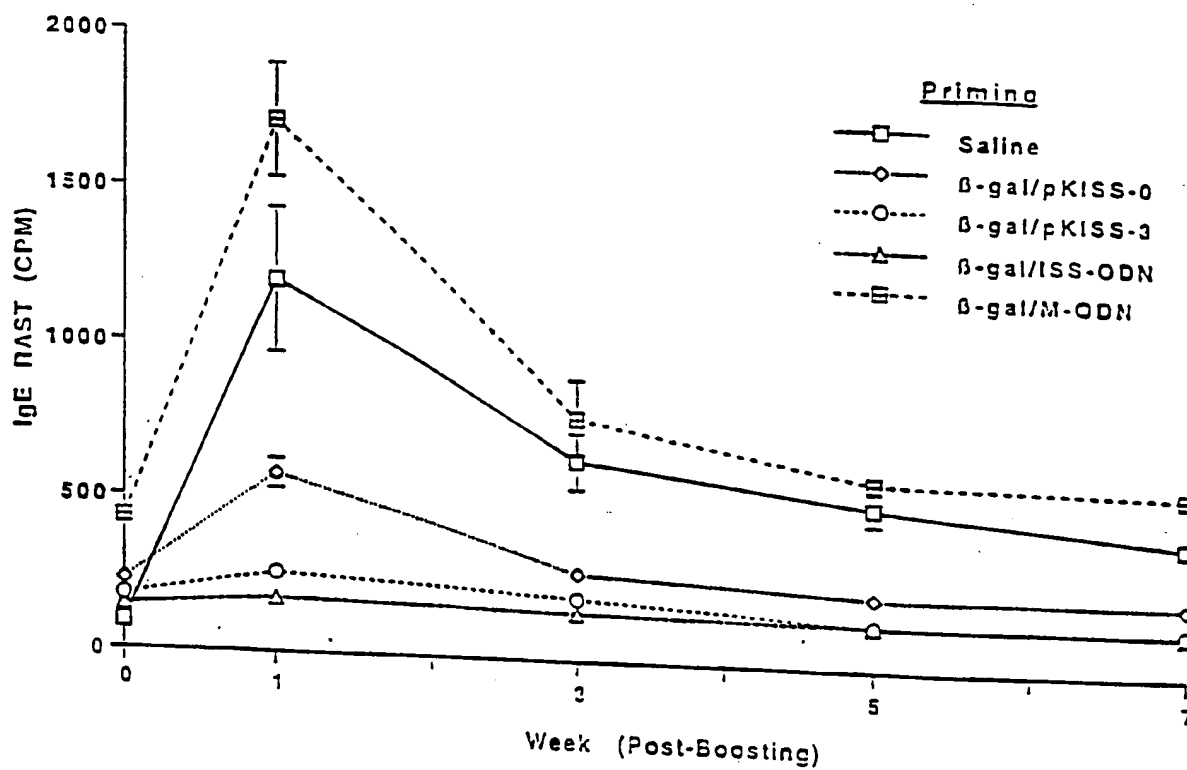


FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11391**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12N 15/00, 15/63, 15/79, 15/09; A61K 48/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 6, 69.1, 172.3; 514/44; 935/62, 55, 71, 65; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CAPLUS, MEDLINE, STN, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YAMAMOTO et al. Mode of Action of Oligonucleotide Fraction Extracted from Mycobacterium Bovis BCG. Kekkaku. 1994, Vol. 69, No. 9, pages 571-574, see entire article.	1-11, 13-29
Y	YAMAMOTO et al. Unique Palindromic Sequences in Synthetic Oligonucleotides are Required to Induce INF and Augment INF-mediated Natural Killer Activity. The Journal of Immunology. 15 June 1992, Vol. 148, No. 12, pages 4072-4076, see entire article.	1-11, 13-29
Y	XU et al. Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein, gp63, of L. major. Immunology. 1995, Vol. 84, pages 173-176, see entire article.	1-11, 13-29



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

10 SEPTEMBER 1998

Date of mailing of the international search report

14 OCT 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11391

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEDEGAH et al. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. Proceedings National Academy of Sciences, USA. October 1994, Vol. 91, pages 9866-9870, see entire article.	1-11, 13-29
Y	ULMER et al. Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein. Science. 19 March 1993, Vol. 259, pages 1745-1748, see entire article.	1-11, 13-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11391**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 12
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/11391

A. CLASSIFICATION OF SUBJECT MATTER:

US CL : 435/320.1, 6, 69.1, 172.3; 514/44; 935/62, 55, 71, 65; 536/23.1, 24.5